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TPA induces repression of EGF receptor gene expression

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Epidermal growth factor receptor gene expression in response to the tumor promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was analyzed in the A549 human lung adenocarcinoma cell line using DNA transfection. After 8 h of treatment a definite repression (45%) of EGF receptor gene transcription could be seen. Repression was maintained for up to 24 h. Little or no induction of transcription could be seen prior to the onset of repression for potential enhancer regions both upstream and downstream of the translation start site. The *cis*-element(s) responsible for repression were localized between residues –384 and –151 of the EGF receptor gene promoter region using a deletion plasmid series.

Epidermal growth factor receptor; 12-*O*-Tetradecanoylphorbol-13-acetate; Repression of transcription; GCF

1. INTRODUCTION

Epidermal growth factor (EGF) interacts with the EGF receptor to stimulate growth and proliferation of a variety of cells in vitro and in vivo [1,2]. The EGF receptor gene is homologous to the product of the v-*erb* B oncogene [3] and over-expression of normal EGF receptors on the cell surface via expression vector transfection has been shown to transform Swiss 3T3 cells in an EGF dependent manner [4,5]. Recently, we showed that squamous cell carcinomas of esophagus and lung have significantly higher levels of EGF receptors in primary tumor tissue than adjacent tissue or other carcinomas [6,7]. Furthermore, we suggested that the increase in receptor number plays an important role in the growth of these tumors as well as their malignancies [8]. These data indicate that strict regulation of EGF receptor gene expression is critical for normal cell growth. In an effort to ascertain mechanisms of this regulation we have looked at the transcriptional response of the EGF receptor gene to the tumor promoter TPA in the A549 human lung adenocarcinoma cell line using DNA transfection analysis. We find that the EGF receptor gene is repressed after exposure to TPA with little or no evidence of induction. The *cis*-elements responsible for this repression were localized. These results are discussed in light of other recent findings.

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Abbreviations: EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMSO, dimethyl sulfoxide; CAT, chloramphenicol acetyltransferase

2. MATERIALS AND METHODS

2.1. *Cell maintenance and TPA treatment*

A549 cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and RPMI 1640 supplemented with 10% fetal bovine serum, penicillin and streptomycin. Cells were treated for the indicated times with TPA (Sigma), dissolved in DMSO at a final concentration of 20 ng per ml of media. Control cells were treated with DMSO only for equal amounts of time.

2.2. *Plasmid construction*

The insert of pERCAT-I [9] containing 1.1 kb of EGF receptor gene regulatory region was isolated after *Hin*dIII restriction digest. It was subsequently digested with *Taq*I, *Dde*I, *Ava*II and *Bgl*I separately and relevant fragments were isolated. Fragments were blunt-ended with Klenow fragment or T4 DNA polymerase followed by *Hin*dIII linker ligation using T4 DNA ligase. Resulting fragments were introduced into the pSVOCAT vector *Hin*dIII site [10]. Recombinants were screened for correct orientation by restriction enzyme mapping. Plasmids containing additional regulatory domains were constructed by similar methods. pERCAT-Ex2.2 was created by isolating an *Eco*RI-*Sac*I 2.2 kb fragment from pEP5 which contains a 5.2 kb genomic insert as described [10]. The insert was *Xho*I linkered and cloned into an *Xho*I linkered *Hin*dIII site in pSVOCAT. pERCAT-Int2.2 contains a 2.2 kb *Pvu*II-*Pvu*II insert also derived from pEP5. The 2.2 kb insert was *Bam*HI linkered and cloned into the *Bam*HI site of pERCAT-I.

2.3. *DNA transfection and CAT assay*

Deletion plasmids were transiently transfected into A549 cells as described previously [11]. Briefly, 6×10^5 cells were seeded in 10 cm tissue culture dishes 20 h prior to transfection. 7 μg of CAT plasmid and 3 μg of a plasmid containing the β-galactosidase gene (pCH110) [12] as an internal control were transfected per plate. Five h post transfection the cells were glycerol shocked. Cells were then incubated in normal growth media for 48 h after which cells were lysed with three freeze-thaw cycles. CAT [11] and β-gal [12] assays were performed as previously described.

3. RESULTS

To clarify the transcriptional effects of TPA on EGF receptor gene expression, DNA transfection analysis

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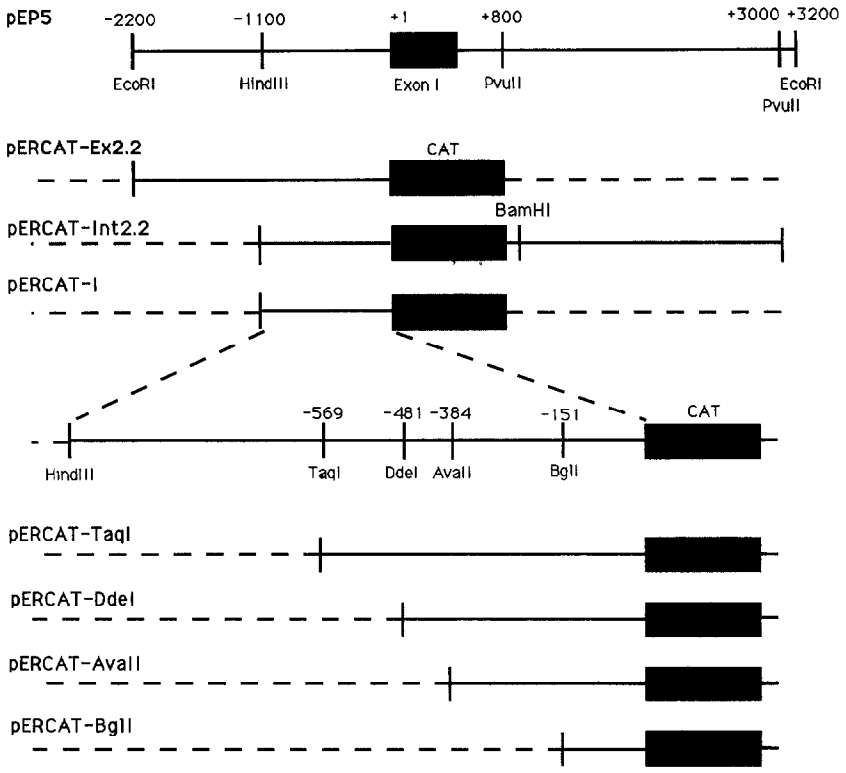

Fig. 1. Structure of EGFR-CAT plasmids. Plasmids were created as described in Materials and Methods. Thin solid lines represent regulatory DNA contained within a given plasmid. Dashed lines represent pSVOCAT sequences.

was carried out using plasmids containing regulatory regions of the EGF receptor gene (Fig. 1). Time course analysis with pERCAT-I revealed repression of EGF receptor gene expression. This repression could not be seen until approximately 5 h after TPA treatment; reaching a maximum by 8 hours and staying constant up to 24 h (Fig. 2). At no point could significant induction of transcription be seen.

This lack of induction in response to TPA was surprising because steady-state EGF receptor mRNA accumulation in A549 cells seems highly sensitive to TPA. Previous results showed an approximate 10 fold increase in EGF receptor mRNA in Northern blot analysis after 4–6 h of TPA treatment. Additional experiments suggested a transcriptional mechanism responsible for this induction (S. Gamou, unpublished observations). To test the theory that *cis*-elements responsive to TPA residue outside of the region tested, we examined two additional potential regulatory regions. Plasmid pERCAT-Ex2.2 contains an additional 1.1 kb of DNA directly upstream of that contained within pERCAT-I. pERCAT-Int2.2 contains 2.2 kb of DNA from the first intron (Fig. 1). Both regions have been suggested to contain enhancer elements [12]. As shown in figure 3a, no induction of CAT activity could be seen with either of the plasmid constructs after 4 h of TPA treatment which coincides with the point of maximum mRNA accumulation. These results suggest either that TPA induced expression of EGF receptor mRNA occurs by a mechanism not involving an increase in transcription or that the *cis*-element conferring transcriptional responsiveness to TPA is not contained within any of the plasmids thus far examined. Experiments are currently underway to address this question.

To localize the *cis*-elements required for repression, deletion plasmids were constructed from pERCAT-I as shown in Fig. 1. pERCAT-I exhibited 43% repression after 16 h TPA exposure (Fig. 3b). This value was quite constant for a number of deletion plasmids as shown in

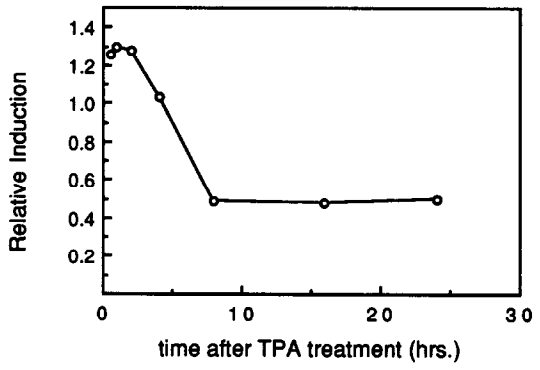

Fig. 2. CAT analysis: time course. Induction or repression was determined by comparison with DMSO control for each time point included. The results are typical of numerous trials.

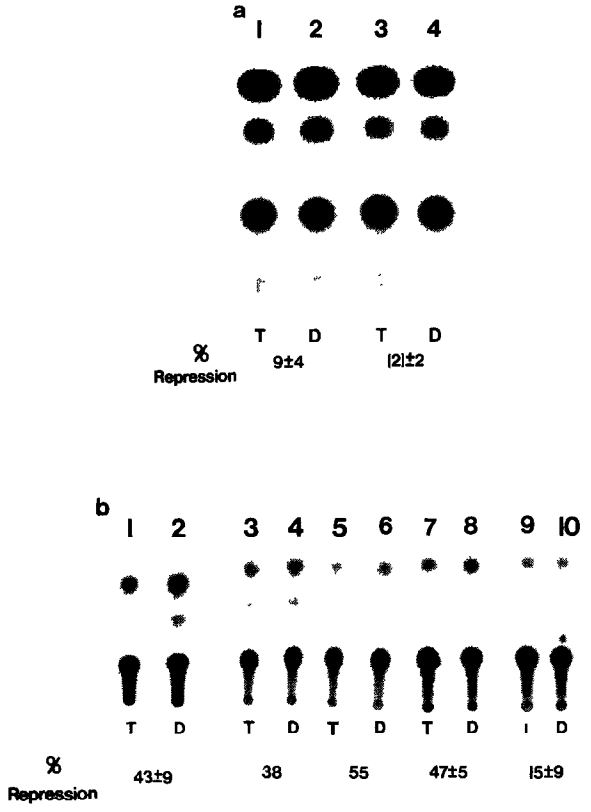

Fig. 3. (a) CAT analysis: pERCAT-Ex2.2 and pERCAT-Int2.2. Assays were done in triplicate with standard error shown. %CAT activity was determined comparing cells transfected and treated with TPA for 4 h vs cells transfected and treated with DMSO. 1, 2: pERCAT-Int2.2. 3, 4: pERCAT-Ex2.2. 'T' corresponds to TPA treated cells and 'D' corresponds to DMSO treated cells. (b) CAT analysis: deletion plasmids. Assays were done in triplicate with standard error shown except for pERCAT-*TaqI* and pERCAT-*DdeI* which are the results of a single experiment. Transfected wells were treated with TPA for 16 h and compared to control cells treated with DMSO for an equal amount of time. 1, 2: pERCAT-I; 3, 4: pERCAT-*TaqI*; 5, 6: pERCAT-*DdeI*; 7, 8: pERCAT-*AvaII*; 9, 10: pERCAT-*BglI*.

Fig. 3b including pERCAT-AvaII which contains only 384 bp of upstream DNA and exhibited 47% repression. The deletion plasmid containing 5'DNA from −16 to −151, however, showed only 14% repression after 16 h TPA treatment. These results indicate that the major *cis*-element(s) required for TPA induced repression reside with a 230 bp sequence between −384 and −151.

4. DISCUSSION

A number of factors including TPA, EGF and retinoic acid can cause an increase in EGF receptor mRNA accumulation in a variety of cell lines [13–17]. The mechanism responsible for this accumulation is unclear. Clark et al. suggest that treatment of A431 cells with EGF leads to enhanced mRNA stability [14]. Others, however, suggest that the increase in mRNA seen after EGF treatment of MDA468 cells could not be due to mRNA stability alone [17]. In our study, A549 cells transiently transfected with up to 3.3 kb of EGF receptor gene regulatory region showed no significant induction of transcription after TPA treatment. However, repression of transcription could clearly be seen with a maximum repression at 8 h. This time closely approximates the point of maximum EGF receptor mRNA accumulation in MDA468 human breast cancer cells after TPA treatment [17].

Deletion analysis localized the major *cis*-element conferring repression between residues −384 and −151. This region has been characterized for a number of regulatory proteins including GCF, a DNA binding protein recently shown to repress EGF receptor gene transcription [18]. We speculate the GCF may be responsible for the repression of EGF receptor gene transcription after TPA treatment. Two binding sites for GCF occur within the region −384 to −151. Additionally, one weak binding site occurs between −90 and −151 which may be responsible for the low level of repression seen with the −16 to −151 plasmid.

These results suggest a complex regulatory response of the EGF receptor gene to TPA involving both positive regulation perhaps by mRNA stabilization and negative regulation at the level of transcription via the putative activity of GCF. Such complexity may be necessary to modulate the response to various mitogens including TPA and EGF.

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